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Research Paper

Oligosaccharidic fractions derived from *Triticum vulgare* extract accelerate tissutal repairing processes in in vitro and in vivo models of skin lesions

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ABSTRACT

Ethnopharmacological relevance: Triticum vulgare has been extensively used in traditional medicine thanks to its properties of accelerating tissue repair. The aqueous extract of *Triticum vulgare* (TVE) is currently an active component used by Farmaceutici Damor in the manufacture of certain pharmaceutical products already marketed in Italy and abroad under the brand name Fitostimoline®, in the formulation of cream and medicated gauze and is commonly used for the treatment of decubitus ulcers, sores, burns, scarring delays, dystrophic diseases, and, more broadly, in the presence of problems relating to re-epithelialization or tissue regeneration. The active components of Fitostimoline®-based products determine a marked acceleration of tissutal repairing processes, stimulate chemotaxis and the fibroblastic maturation, and significantly increase the fibroblastic index, which are crucial points in the repairing processes. The aim of the present paper was to identify and characterize the active fractions of TVE responsible for the pharmacological effect in tissutal repairing processes.

Materials and methods: Several fractions obtained from TVE by ultrafiltration procedures and HPAE chromatography were tested to measure their growth-enhancing activity on NIH-3T3 fibroblasts. The healing action of the same fractions, prepared as cream formulation, was assessed in rat subjected to two different models of skin lesion, skin scarification and excision.

Results: Our results showed a pro-proliferative effect of the fractions ST-98 and $K > 1000$ in NIH-3T3 fibroblasts. Moreover these fractions formulated as cream preparations were effective also in in vivo models of skin lesion.

Conclusions: The results of the present study showed that these active fractions of TVE are responsible for its pro-proliferative effect.

1. Introduction

In recent years one of the main aims of biological and clinical research has been the isolation and identification of factors which facilitate and enhance normal wound healing processes in animals and humans. The presence in mammalian cells of specific factors responsible for accelerating the healing process (Ten Dijke and Iwata, 1989) and the existence of compounds within plants such as *Centella asiatica* and *Gardenia jasminoides* Ellis fructus (Morisset et al., 1987), still only partially identified, with these pharmacological properties have been well established. The aqueous extract of *Triticum vulgare* (TVE) obtained from the whole germinated plant and containing mainly oligosaccharidic components has different biological properties including a mitogenic effect both in plants and in the mouse fibroblast line BALB/c NIH-3T3 (Farinella et al., 1986; Favit et al., 1992), useful in the process of wound healing.

Moreover the aqueous extract of *Triticum vulgare* is currently an active component used by Farmaceutici Damor in the manufacture of certain pharmaceutical products already marketed in Italy and abroad under the brand name Fitostimoline®, in the formulation of

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cream and medicated gauzes and is commonly used for the treatment of decubitus ulcers, sores, burns, scarring delays, dystrophic diseases, and, more broadly, in the presence of problems relating to re-epithelialization or tissue regeneration (Chaturvedi et al., 2010; Martini et al., 2011; Serafini and Saponati, 2012).

Triticum vulgare has been extensively used in traditional medicine thanks to its properties of accelerating tissue repair. In particular, scientific evidence documented that the wheat sprout oil has been used in traditional Iranian medicine for dermotonic and skin beauty, face freckles, moisturizing and repair the minute pores of the face skin (Mikaili et al., 2012).

The active components of Fitostimoline®-based products determine an acceleration of tissutal repairing processes, stimulate chemotaxis and the fibroblastic maturation, and significantly increase the fibroblastic index, which are crucial points in the repairing processes (Viano and Santiano, 1978; Fiore et al., 1993). It has been suggested that these activities are due to the accelerated protein synthesis and to the enhanced ability of captation and incorporation of marked proline from tissues (Viano and Santiano, 1978; Fiore et al., 1993; Vanden Berghe et al., 1993).

With the aim of identifying and characterizing the active fractions of TVE responsible for its pharmacological effect, in the present study we investigated the effect of several fractions obtained from TVE on the growth of mouse fibroblast NIH-3T3 cells, that have been chosen as an appropriate in vitro experimental model for our studies since TVE has been shown to enhance the growth of mouse fibroblast NIH-3T3 cells (Farinella et al., 1986). For this purpose the fractions have been isolated by ultrafiltration procedures and HPAE chromatography and their growth-enhancing activity on NIH-3T3 fibroblasts has been determined and compared with those of the crude TVE and fetal bovine serum (FBS). Here we report that the effect of some fractions on the growth of mouse fibroblast NIH-3T3 cells assessed by proliferation assays and scratch tests is comparable to that of the whole aqueous extract of *Triticum vulgare*.

Furthermore, we have investigated the healing action of these in vitro active fractions in two different in vivo models of skin lesion, skin scarification and excision in rats. The healing action was evaluated 3 or 7 days after skin damage induction.

2. Materials and methods

2.1. Plant description

Triticum vulgare, the binomial scientific name of a plant of Graminaceae family, is the commonly known wheat plant. It is grown under controlled conditions in the laboratory of Farmaceutici Damor, Naples, Italy. The voucher specimen is DF/237/2014 and is deposited in the herbarium of the Medical Botany Chain of University of Salerno, Italy. The commercially available seeds are purchased from Consorzio Agrario Lombardo Veneto from Northern Italy. The batch number for the seeds used for the present paper was 12/001-B10148/201/04

2.2. Cell cultures

Mouse embryonic fibroblasts BALB/c NIH-3T3 (American Type Culture Collection, Rockville, MD) were maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich S.r.l., Milan, Italy) and 1% Penicillin–Streptomycin–Glutamine (Sigma). NIH-3T3 were grown on 100 mm-ø Falcon culture plates at 37 °C in a humidified atmosphere containing 5% CO_2, and sub-cultured every 2–3 days. Confluent cultures were trypsinized with 2 ml of trypsin (6 g trypsin (for 1 L), 0.2 M EGTA (ethylene glycol tetraacetic acid), 5.5 mM glucose, make up to volume with PBS 1X, pH 7.2–7.4) at 37 °C. To inactivate trypsin, 4 ml of DMEM was added and the pellet obtained after centrifugation was diluted with fresh medium to an appropriate plating density.

2.3. Test samples preparation and storage

Triticum Vulgare extract (TVE) and the different fractions obtained from the extract (as described in Section 3) were filtered through a 0.2 μm membrane filter (Millipore, Billerica, MA) for sterilization and stored at 4 °C. Fetal bovine serum (FBS) (Sigma-Aldrich S.r.l., Milan, Italy) was stored at −20 °C. PBS 1X (PBS 10X: 1.37 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 19 mM KH_2PO_4), H_2O and H_2O pH 4.0 were filtered through a 0.2 μm membrane filter for sterilization and stored at 4 °C. K0901 and K0801 are two batches of *Triticum vulgare* extract prepared according to standard manufacturing protocol of germination, extraction and purification described in Common Technical Document (CTD) and filed at Italian Medicines Agency (AIFA) by Farmaceutici Damor. For each 1 kg of germinated seeds, 120 L of aqueous extract of *Triticum vulgare* is produced.

ST-98 is a purified fraction of oligosaccharides obtained by semi-preparative HPAE chromatography as described in Section 3.1. It is present at 4% concentration in standard industrial batch of TVE. All the researchers were blinded to the treatments.

2.4. Sugar analysis by HPAE chromatography after acid hydrolysis

Sugar analysis of both K > 1000 and ST-98 fractions was performed according to the following protocol. Trifluoroacetic acid (TFA) 2 M (0.5 ml) is added to the dried test fraction (100 μg) in a PIERCE vial. The vial is placed under vacuum and then placed in a thermostated Reactiterm (PIERCE) apparatus at 121 °C for 4 h, at the end of this period the vial is cooled, opened and the content is freeze-dried and then washed thrice with water to completely remove the TFA residues. The dried sample is redissolved in 20 μl of water and injected on DIONEX Carbopac PA1 column, using as an eluent system a linear binary gradient of 16 mM aqueous NaOH (eluent A) and 200 mM aqueous NaOH (eluent B). The column is initially equilibrated with 100% of eluent A at a flow of 1 ml/min; after injection a linear gradient rising up to 100% of eluent B in 20.2 min is applied. The chromatographic peaks are identified by comparison with monosaccharides reference standard solutions (DIONEX). HPAE chromatography coupled to PAD is a fast and reliable chromatographic system particularly indicated for carbohydrates determination. The procedure has been setted up using a DIONEX system consisting of a modular high resolution liquid chromatography equipped with a double piston pump system, a non-stop helium base degassing system, a programmable gradient generator and an automatic injection valve. The detection system consists of an amperometric pulsed (PAD) DIONEX detector and an ALTEX recorder. The used column is an ion exchange Carbopac PA1 DIONEX (30 × 0.46 cm^2).

2.5. Mass spectrometry

Mass spectra were obtained by either MALDI Micro MX-Waters or QTOF Premiere-Waters instruments under the following experimental.

MALDI TOF MS analysis has been carried out in linear positive mode; the laser energy has been set to the arbitrary value of 250. The samples were diluted in water/acetonitrile with 0.1% TFA. The mass range was set between 500 and 10,000 *m/z*. Calibration has been carried out using enolase peptide mixture (Waters Co.) between 500 and 10,000 *m/z*. Different matrices were initially tested: dihydroxybenzoic acid (DHB), alfa-ciano-4-hydroxycinnamic acid (CHCA), synapinic acid (SA) and 2,4,6-trihydroxyacetophenone (THAP). The

best spectral quality was obtained with THAP, which was then used as a standard matrix for all the spectra.

ESI-QTOF-MS analysis has been carried out in negative mode and all mass spectra were acquired between 500 and 3500 *m*/*z*; the samples were diluted in water/30% 2-propanol and 2% of triethyl-amine. Each multi-charged ions spectrum has been deconvoluted using the WATERS Co. software MassLynk 4.0. Calibration has been carried out using NaI solution.

2.6. Cell growth assays

The growth-enhancing activity of the aqueous extract of *Triticum Vulgare* (TVE) and of the fractions obtained from the extract was tested on fibroblasts made quiescent by growth in low concentrations of fetal bovine serum (FBS). Cells were plated at a density of 3×10^4–4×10^4/well in Falcon culture six-wells plates in DMEM supplemented with 10% FBS. The medium was renewed after 24 h and replaced with fresh medium containing 0.6% FBS; the cells being allowed to grow for an additional 48 h before the addition of drugs (added a volume of substance to DMEM equal to 10%), H_2O and H_2O pH 4.0, PBS 1X (PBS 10X: 1.37 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 19 mM KH_2PO_4) (added a volume of substance to DMEM equal to 10%) and FBS (medium replaced with DMEM supplemented with 10% fetal bovine serum). Drugs, H_2O, H_2O pH 4.0, PBS 1X and serum were added only once throughout the growth period. After 48 h, cell number was estimated on triplicate samples thrice by trypsinizing the cultures and counting the cells with a cell counting chamber Neubauer (0.1 mm); cells were stained with Trypan Blue (Sigma-Aldrich S.r.l., Milan, Italy) dye before counting.

2.7. Scratch test

The growth-enhancing activity of the drugs was also evaluated through the scratch test. Cells were plated at a density of 25×10^4–35×10^4/plate in 100 mm-ø Falcon culture plates in DMEM supplemented with 10% FBS. The medium was renewed after 24 h and replaced with fresh medium containing 0.6% FBS; the cells being allowed to grow for an additional 48 h before the scratch followed by the addition of drugs (added a volume of substance to DMEM equal to 10%), H_2O and H_2O pH 4.0, PBS 1X (PBS 10X: 1.37 M NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 19 mM KH_2PO_4) (added a volume of substance to DMEM equal to 10%) and FBS (medium replaced with DMEM supplemented with 10% fetal bovine serum). Drugs, H_2O, H_2O pH 4.0, PBS 1X and serum were added only once throughout the growth period. Cell growth was observed under an inverted microscope (Zeiss, Arese, Milan, Italy) after 24 and 30 h. The growth-enhancing activity of the drugs was evaluated in triplicate for three times.

2.8. Experimental in vivo groups

Male Sprague–Dawley rats (Charles River, Calco, Lecco, Italy) weighing 250–300 g were housed under diurnal lighting conditions (12 h darkness/light). Experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of "Federico II", University of Naples, Italy.

2.9. Chemicals and drugs

The commercial product Fitostimoline® cream (Damor Pharmaceutics, Naples, Italy), which contains as active principle the TVE, was used as positive control, whereas the vehicle was used as negative control. The fractions used demonstrated their efficacy in in vitro assays. The fractions ST-98, K0901, $K > 1000$ were prepared at 10% concentration as cream using the same excipients as the commercial cream. As negative controls, a cream containing only the excipients without *Triticum vulgare* extract was used.

2.10. Skin scarification model

Rats, anaesthetized using 1.5% sevofluorane, 70% N_2O, and 28.5% O_2, were shaved on the dorsal region, and thereafter an area of about 0.5 cm in diameter was scarified. Subsequently, the area was medicated by the application of the vehicle, or Fitostimoline® cream (Damor Pharmaceutics, Naples, Italy) as positive control, or each of the fractions in a cream pharmaceutical form. The application was repeated daily, and the wound covered with sterile gauze was fixed with bandage. The process of wound healing was assessed by the measurement of the scar after 3 days from the beginning of the application. At the end of the experiment the animals were sacrificed by decapitation after anesthesia. Eight rats were used for each experimental group.

2.11. Skin excision model

Rats, anesthetized using 1.5% sevofluorane, 70% N_2O, and 28.5% O_2, were shaved on the dorsal region, and thereafter the area of excision was delimited by the use of methylene blue. The area was approximately 2 cm^2. The lesion was performed by using scalpels and scissors. Subsequently, the area has been medicated by the application of the vehicle as negative control, or Fitostimoline® cream (Damor Pharmaceutics, Naples, Italy) as positive control, or each of the fractions in a cream pharmaceutical form. The application was repeated daily, and the wound covered with sterile gauze was fixed with a bandage. The process of wound healing was assessed by measuring the area of the wound 7 days from the beginning of the application. At the end of the experiment the animals were sacrificed after anesthesia. Eight rats were used for each experimental group.

2.12. Statistical analysis

Data are expressed as mean ($\pm$ SEM). Statistical comparisons between controls and treated experimental groups were performed using the one-way ANOVA, followed by Newman Keul's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and chromatographic profiles of tested fractions

Two batches of *Triticum vulgare* extract (TVE K0801 and K0901) were prepared according to standard manufacturing protocol at Farmaceutici Damor. Successively, to obtain 2 whole fractions with different molecular weights, 1 L of TVE K0901 was submitted to molecular weight fractionation through a standard ultrafiltration procedure in an Amicon 200 ml ultrafiltration cell equipped with a Millipore NMWL 1000 Da membrane of 63.5 mm (2 bar N_2 pressure). The concentrated fraction (labeled $K > 1000$), containing an oligosaccharide mixture with a nominal MW higher than 1000 Da, was recovered from top of the filter membrane after thoroughly washing with distilled water. The solution eluted from the filter membrane, containing lower MW components, was labeled as $K < 1000$. Both fractions were diluted to the same volume that of the starting sample.

In order to verify the efficiency of the adopted fractionation procedure, starting solution (K0901) and ultrafiltration fractions ($K < 1000$ and $K > 1000$) were submitted to analytical HPAE (High Pressure Anion Exchange) chromatography, using a PAD (Pulsed Amperometric Detector), that is particularly indicated for carbohydrates analysis. The low molecular weight oligosaccharides are eluted at lower retention times with respect to oligosaccharides with higher molecular weight. The chromatogram of the whole TVE-K0901 batch is shown in Fig. 1A. The TVE-K0801 had a similar HPAE behavior. The chromatograms obtained for $K > 1000$ and

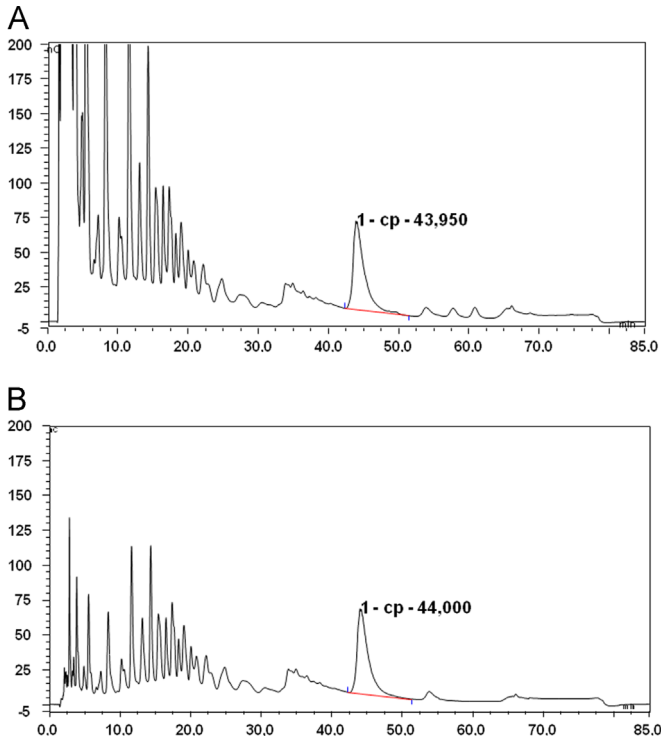

Fig. 1. Chromatographic profiles of tested fractions. Panel A: High Pressure Anion Exchange profile of K0901; and Panel B: High Pressure Anion Exchange profile of K > 1000 fraction. In order to verify the efficiency of the adopted fractionation procedure, starting solution (K0901) and ultrafiltration fractions (K < 1000 and K > 1000) were submitted to analytical HPAE (High Pressure Anion Exchange) chromatography, using a PAD (Pulsed Amperometric Detector), that is particularly indicated for carbohydrates analysis. The chromatography was performed using a linear gradient elution system at high pH, with 0.5 M NaOH (as eluent 1) and 1 M Sodium acetate trihydrate in 0.5 M NaOH (as eluent 2).

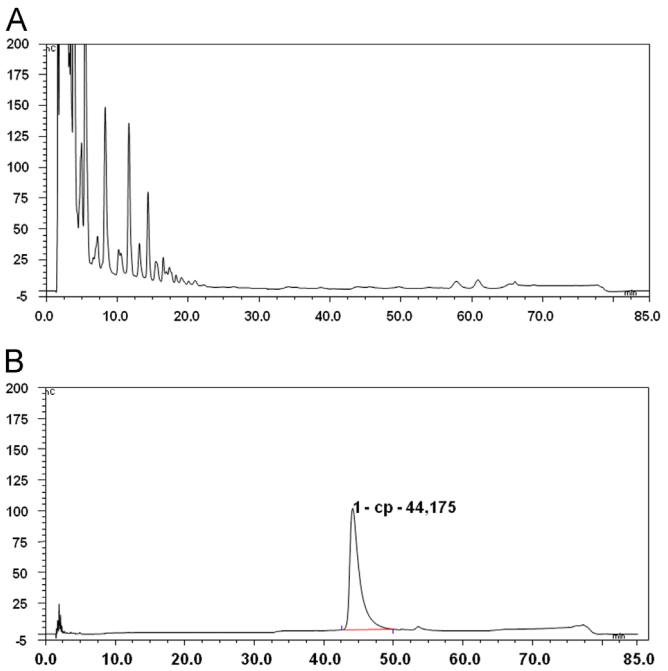

Fig. 2. Chromatographic profiles of tested fractions. Panel A: High Pressure Anion Exchange profile of K < 1000 fraction; and Panel B: High Pressure Anion Exchange profile of ST98 fraction. Analytical HPAE (High Pressure Anion Exchange) chromatography, using a PAD (Pulsed Amperometric Detector), was applied. The chromatography is performed using a linear gradient elution system at high pH, with 0.5 M NaOH (as eluent 1) and 1 M Sodium acetate trihydrate in 0.5 M NaOH (as eluent 2).

K < 1000 fractions are reported in Figs. 1B and 2A, respectively. To prepare ST98 fraction, K > 1000 was submitted to semipreparative separation by HPAE chromatography (at the same conditions above described for the analytical test) in order to isolate the major peak eluting between 42 and 52 min, to be considered as a standard reference. The obtained chromatogram is shown in Fig. 2B. Four more test fractions were prepared by separate extractions – at the same extraction conditions used for the whole extract TVE – of the aerial part (fraction S-M), and of the roots (fraction S-N) of the germinating plants, and also of the germinating soil (fraction S-P) and of the ungerminated seeds (fraction S-S). The chromatograms of these four fractions appeared non-significant.

3.2. Effects of various concentrations of Triticum vulgare extract (TVE) and of different fractions obtained from the extract on cell growth in mouse embryonic fibroblasts BALB/c NIH-3T3

Since *Triticum vulgare* extract (TVE) is widely used for wound healing, and since wound repair requires cell proliferation during the inflammation phase (Ten Dijke and Iwata, 1989), and TVE has been shown to enhance the growth of mouse fibroblast NIH-3T3 cells (Farinella et al., 1986), we studied the influence of TVE and several fractions obtained from TVE on the proliferation of mouse fibroblast NIH-3T3 cells.

Stimulation of cell growth was evaluated after the addition of different amounts of TVE (Fig. 3B) or after the addition of several fractions obtained from TVE (added a volume of substance to DMEM equal to 10%) (Figs. 4B, and 5B) to cell cultures in DMEM with 0.6% fetal bovine serum (FBS) and counting the cells after 48 h of incubation. Starting with 3×10^4–4×10^4 cells/well in Falcon culture six-wells plates a confluent monolayer containing about 1×10^6 cells/well was usually obtained after 5 days in DMEM with 10% FBS. With 0.6% FBS (control), however, cells were still alive after 5 days but no multiplication had taken place. The growth-enhancing activity of the aqueous extract of *Triticum vulgare* (different amounts) (Fig. 3A) and of the different fractions (added a volume of substance to DMEM equal to 10%) (Figs. 4A, and 5A) was evaluated by the scratch test, observing the filling of the strip due to cell proliferation after 24 and 30 h of incubation. Starting with 25×10^4–35×10^4 cells/plate in 100 mm-ø Falcon culture plates a semi-confluent monolayer containing about 2×10^6 cells/plate was usually obtained after one day in DMEM with 10% FBS. Performing the scratch at this stage, the complete closure of the strip was observed after 30 h of incubation.

3.3. Effects of different concentrations of Triticum vulgare extract (TVE) on cell growth in mouse embryonic fibroblasts BALB/c NIH-3T3

The stimulation by TVE was thus characterized by an increase of the cell growth compared with the negative controls. As can be seen from Fig. 3A and B, the cell proliferation reflected by the final cell density at the end of 48 h (Fig. 3B), 24 h and 30 h (Fig. 3A) of incubation showed a dose-dependent response in the concentration range of 1–10% TVE. Higher concentrations of TVE (20% and 30%) exhibited a decreasing stimulation of cell compared with the stimulating capacity (data not shown). In the presence of 10% TVE the final cell density was about six times higher than that of the negative control cultures; also the strip into the scratch test is completely closed when compared to negative controls.

3.4. Effects of several fractions obtained from the extract of Triticum vulgare on cell growth in mouse embryonic fibroblasts BALB/c NIH-3T3

At a later stage, we evaluated the stimulating ability of several fractions obtained from TVE in order to identify some of the active

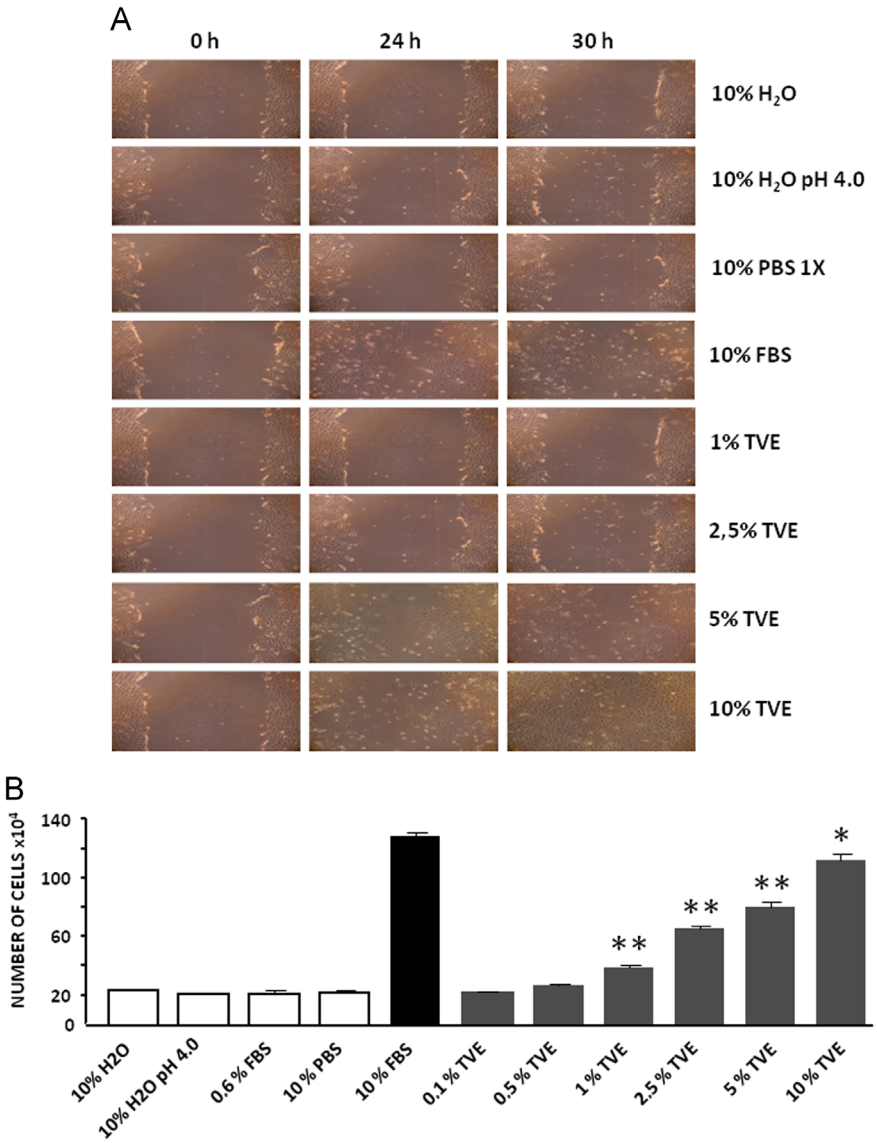

Fig. 3. Effect of different dilutions of TVE on cell proliferation. Panel A: Scratch test: pictures of NIH 3T3 fibroblasts starved with 0.6% FBS at time zero and after 24 h and 30 h by treatment with 10% H_2O, 10% H_2O pH 4.0, 10% PBS 1X, 10% FBS, and different amounts (1%; 2.5%; 5%; and 10%) of TVE. Cells were plated at a density of 25×10^4–35×10^4/plate in 100 mm-ø Falcon culture plates in DMEM supplemented with 10% FBS. The medium was renewed after 24 h and replaced with fresh medium containing 0.6% FBS; the cells being allowed to grow for an additional 48 h before the scratch followed by the addition of drugs (added a volume of substance to DMEM equal to 10%), H_2O, H_2O pH 4.0, PBS 1X (added a volume of substance to DMEM equal to 10%) and FBS (medium replaced with DMEM supplemented with 10% fetal bovine serum). Drugs and serum were added only once throughout the growth period. Cell growth was observed under an inverted microscope (Zeiss) after 24 and 30 h. The growth-enhancing activity of the drugs was evaluated in triplicate for three times. Panel B: Growth-enhancing activity of different amount of the aqueous extract of *Triticum vulgare* (TVE) on NIH 3T3 fibroblasts. Cells were plated at a density of 3×10^4–4×10^4/well in Falcon culture six-wells plates in DMEM supplemented with 10% fetal bovine serum (FBS). After 24 h, cells were starved with fresh medium containing 0.6% FBS for 48 h; subsequently were added to the medium different amounts (0.1–10%) of TVE. Controls with 10% H_2O and H_2O pH 4.0, 10% PBS 1X, 0.6% and 10% FBS were included in the experiment. After 48 h, cell number was estimated by trypsinizing the cultures and counting the cells with a cell counting chamber Neubauer (0.1 mm); cells are stained with the dye Trypan Blue before counting. Average of triplicate for three times was calculated. Each column represents the mean ± SEM of the percentage of increase in cell proliferation. $^*p < 0.05$ versus negative controls (white bars), $^{**}p < 0.05$ versus positive control 10% FBS (black bar).

components of the extract. The fractions were used in experiments to 10%, as the best concentration of TVE. As can be seen from Figs. 4A, B and 5A, B the cell proliferation reflected by the final cell density at the end of the 48 h (Figs. 4B, and 5B), 24 and 30 h (Figs. 4A, and 5A) of incubation showed, for some fractions, an increase comparable to that of the whole extract of *Triticum vulgare*.

In fact, standard TVE extract batches K0801 and K0901 and the fractions ST-98, and K > 1000 (Fig. 4A and B), that contain the major peak eluting between 42 and 52 min (see Figs. 1 and 2), induced cell proliferation approximately seven times higher than negative controls. Moreover the fractions ST-98 and K > 1000 induced an increase in cell proliferation significantly higher than the positive control (FBS) and comparable to the whole extract of *Triticum vulgare* K0801 and K0901. The same fractions induced the complete closure of the strip into the scratch test. Vice-versa, the K < 1000, that does not contain the major peak eluting between 42 and 52 min (see Fig. 2A), appeared to be inactive.

Finally, the fractions S-M and S-N, extracts obtained respectively from the aerial part and by the root of germinating plants of *Triticum vulgare*, as well as the fractions S-P, obtained from the germinating soil, and S-S, extract obtained by ungerminated seeds, showed no proliferative activity on mouse fibroblast NIH-3T3 cells

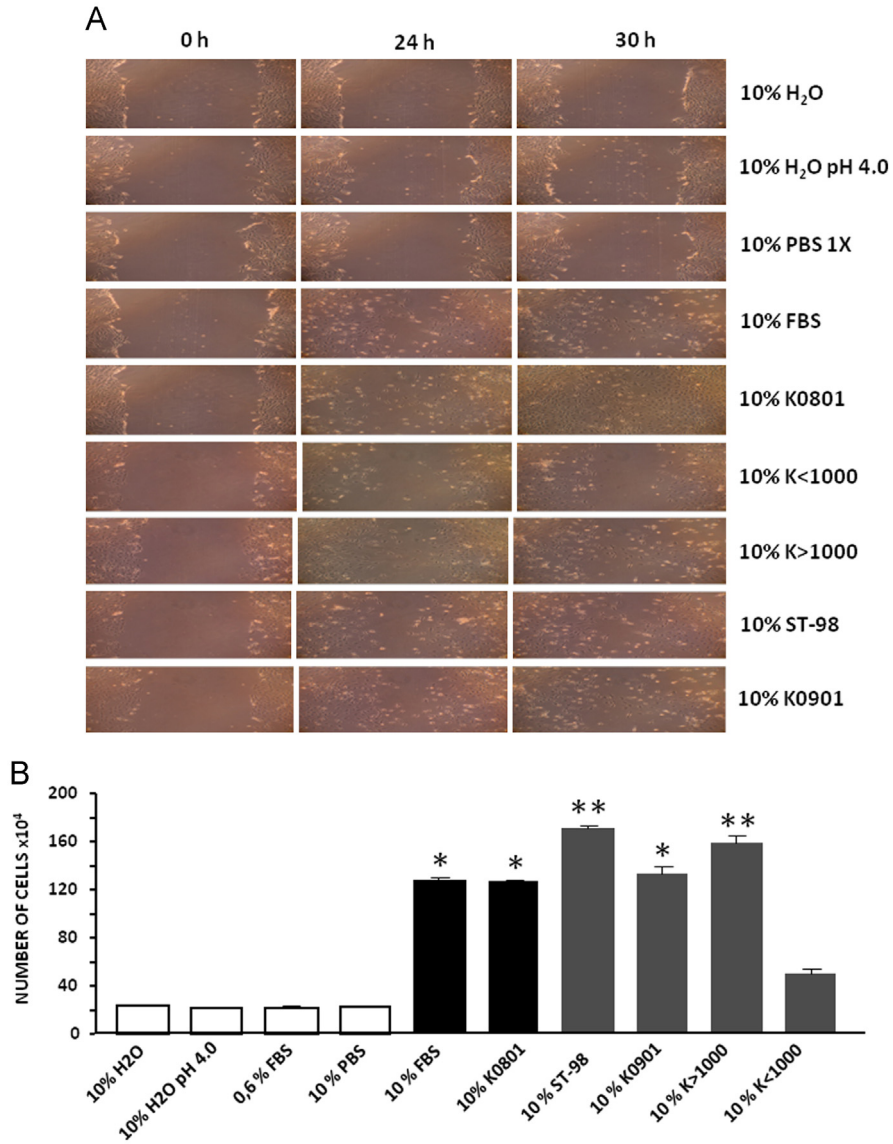

Fig. 4. Effect of fractions ST-98, K0801, K0901, K > 1000 and K < 1000 on cell proliferation. Panel A: Scratch test: pictures of NIH 3T3 fibroblasts starved with 0.6% FBS at time zero and after 24 h and 30 h by treatment with 10% H_2O, 10% H_2O pH 4.0, 10% PBS 1X, 10% FBS, K0801, 10% fractions K < 1000, K > 1000, ST-98 K0801 and K0901. Cells were plated at a density of 25×10^4–35×10^4/plate in 100 mm-ø Falcon culture plates in DMEM supplemented with 10% FBS. The medium was renewed after 24 h and replaced with fresh medium containing 0.6% FBS; the cells being allowed to grow for an additional 48 h before the scratch followed by the addition of drugs (added a volume of substance to DMEM equal to 10%), H_2O, H_2O pH 4.0, PBS 1X (added a volume of substance to DMEM equal to 10%) and FBS (medium replaced with DMEM supplemented with 10% fetal bovine serum). Drugs and serum were added only once throughout the growth period. Cell growth is observed under an inverted Zeiss microscope after 24 and 30 h. The growth-enhancing activity of the drugs was evaluated in triplicate for three times. Panel B: Growth-enhancing activity of different fractions (ST-98, K0801, K0901, K > 1000, K < 1000) obtained from the aqueous extract of *Triticum vulgare* (TVE) on NIH 3T3 fibroblasts. Cells were plated at a density of 3×10^4–4×10^4/well in Falcon culture six-wells plates in DMEM supplemented with 10% Fetal bovine serum (FBS). After 24 h, cells were starved with fresh medium containing 0.6% FBS for 48 h; subsequently was added to the medium a volume of substance (different fractions) to DMEM equal to 10%. Controls with 10% H_2O and H_2O pH 4.0, 10% PBS 1X, 0.6% and 10% FBS and 10% TVE were included in the experiment. After 48 h, cell number was estimated by trypsinizing the cultures and counting the cells with a cell counting chamber neubauer (0.1 mm); cells were stained with the dye Trypan Blue before counting. Average of triplicate for three times was calculated. Each column represents the mean ± SEM of the percentage of increase in cell proliferation. *$p < 0.05$ versus negative controls (white bars), **$p < 0.05$ versus positive controls 10% FBS and 10% TVE (black bars).

when compared to positive control (FBS) and whole extract of *Triticum vulgare* (Fig. 5B). Also as part of the scratch test, the four fractions, that does not contain the major peak eluting between 42 and 52 min, showed no effect on cell proliferation as evidenced by failure to closure of the strip (Fig. 5A).

3.5. Effect of fractions ST-98, K0801, K0901, K > 1000 on healing in the rat model of skin scarification

The results obtained in the model of skin scarification showed that all the tested fractions have a healing power significantly greater than that mediated by the vehicle, when evaluated 3 days after induction of the skin lesion. In particular, the efficacy of the fractions was comparable to that of Fitostimoline® cream already present in the market (Fig. 6).

3.6. Effect of fractions ST-98, K0801, K0901, K > 1000 on healing in the rat model of skin excision

The results obtained in the model of skin excision showed that all the tested fractions have a healing power significantly greater than that mediated by the vehicle, when evaluated 7 days after

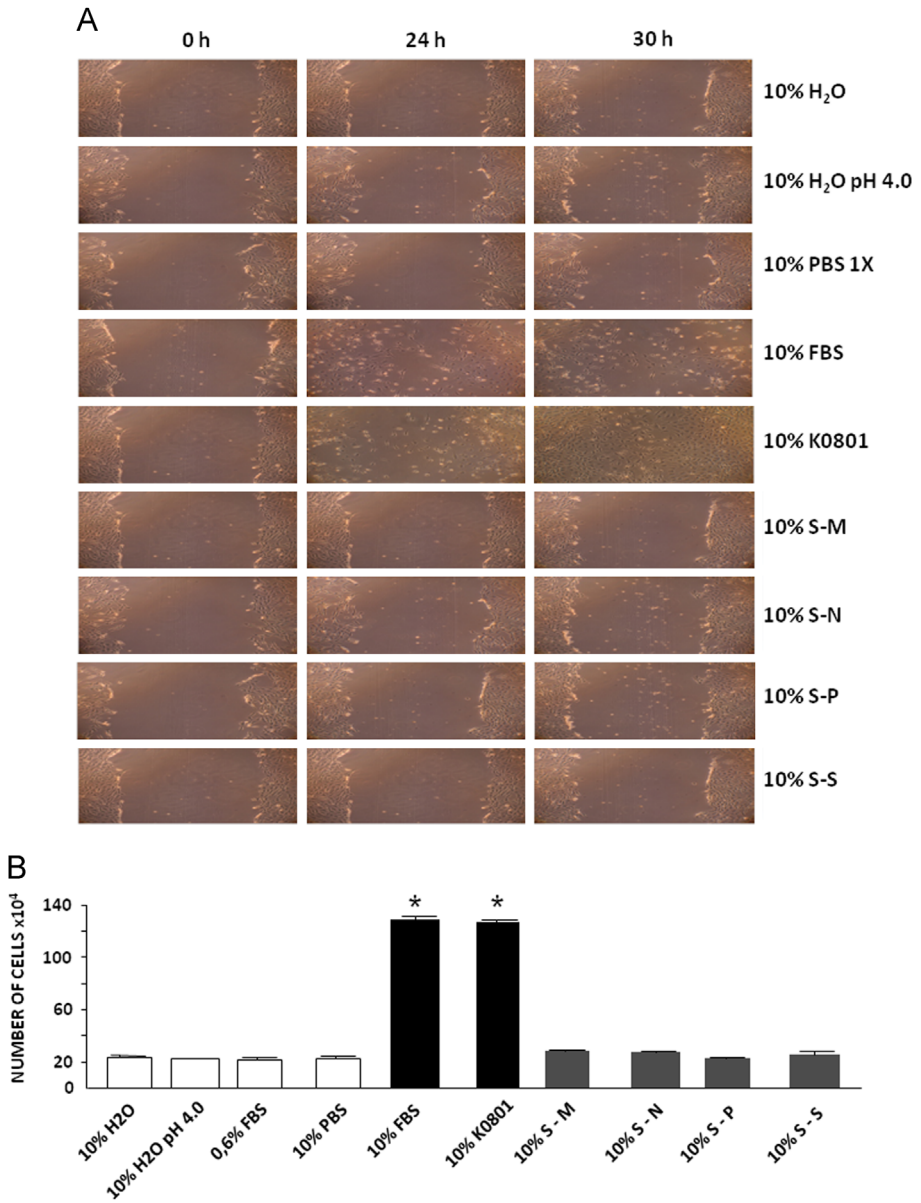

Fig. 5. Effect of group of fractions S-M, S-N, S-P and S-S on cell proliferation Panel A: Scratch test: pictures of NIH 3T3 fibroblasts starved with 0.6% FBS at time zero and after 24 h and 30 h by treatment with 10% H_2O, 10% H_2O pH 4.0, 10% PBS 1X, 10% FBS, K0801, and 10% fractions S-M, S-N, S-P and S-S. Cells are plated at a density of 25×10^4–35×10^4/plate in 100 mm-ø Falcon culture plates in DMEM supplemented with 10% FBS. The medium was renewed after 24 h and replaced with fresh medium containing 0.6% FBS; the cells being allowed to grow for an additional 48 h before the scratch followed by the addition of drugs (added a volume of substance to DMEM equal to 10%), H_2O, H_2O pH 4.0, PBS 1X (added a volume of substance to DMEM equal to 10%) and FBS (medium replaced with DMEM supplemented with 10% fetal bovine serum). Drugs and serum were added only once throughout the growth period. Cell growth was observed under an inverted microscope (Zeiss) after 24 and 30 h. The growth-enhancing activity of the drugs was evaluated in triplicate for three times. Panel B: Growth-enhancing activity of different fractions (S-M, S-N, S-P and S-S) obtained from the plant of *Triticum vulgare* on NIH 3T3 fibroblasts. Cells were plated at a density of 3×10^4–4×10^4/well in Falcon culture six-wells plates in DMEM supplemented with 10% fetal bovine serum (FBS). After 24 h, cells were starved with fresh medium containing 0.6% FBS for 48 h; subsequently was added to the medium a volume of substance (different fractions) to DMEM equal to 10%. Controls with 10% H_2O and H_2O pH 4.0, 10% PBS 1X, 0.6% and 10% FBS and 10% TVE are included in the experiment. After 48 h, cell numbers are estimated by trypsinizing the cultures and counting the cells with a cell counting chamber Neubauer (0.1 mm); cells were stained with the dye Trypan Blue before counting. Average of triplicate for three times is calculated. Each column represents the mean ± SEM of the percentage of increase in cell proliferation. $^*p < 0.05$ versus negative controls (white bars).

induction of the skin lesion. In particular, the efficacy of the fractions was comparable to that of Fitostimoline® cream already present in the market (Fig. 7).

3.7. Data on structural characterization of fractions K > 1000 and ST-98

In order to obtain information on the structural characteristics of the chemical constituents, the fractions K > 1000 and ST-98 were submitted to sugar analysis and Mass spectrometry. This investigation revealed for ST-98 the presence of a discrete series of homologous malto-oligosaccharides with molecular weight ranging from ca. 3800 to 6550 Da, while K > 1000 showed also the presence of minor molecular weight congeners (ca. 1000–4000 Da).

The sugar composition of both fractions K > 1000 and ST-98 was analyzed by HPAE chromatography after acid hydrolysis, according to the experimental protocol described in Section 2.4. The applied procedure allows the identification of all the monosaccharides with the exception of uronic and neuroaminic acids that are decomposed during the acid hydrolysis. For both fractions the only peak revealed in the HPAE chromatogram was identified as glucose, by its retention time and comparison with an authentic standard.

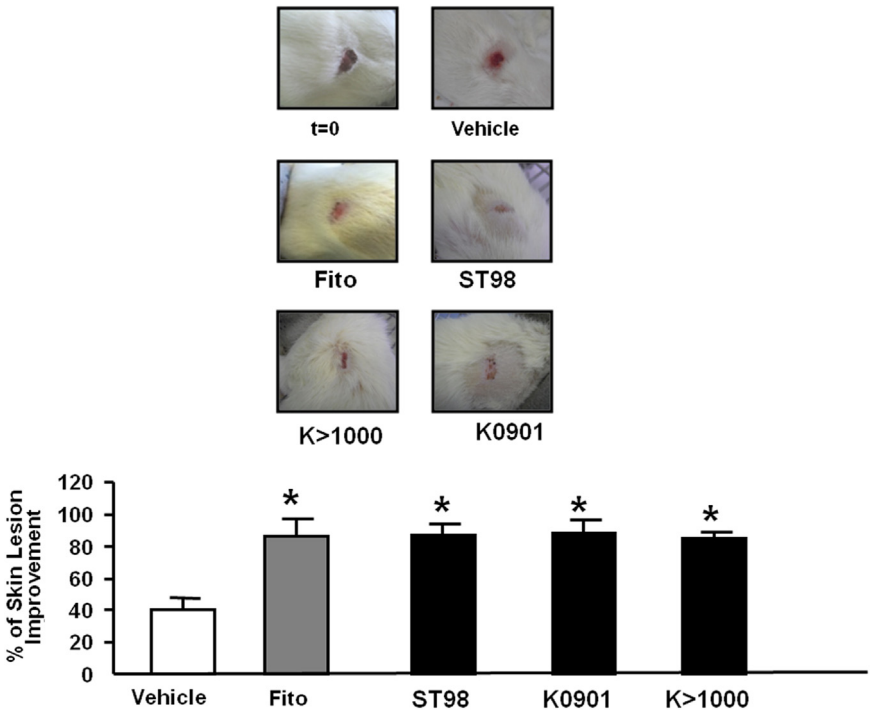

Fig. 6. Effect of fractions ST-98, K0901, K > 1000, on healing in the rat model of skin scarification. The vehicle, or Fitostimoline® cream (Fito), or the fractions ST-98, K0901 and K > 1000 in a pharmaceutical form as a cream, were applied on the lesioned area daily, and the wound covered with sterile gauze was fixed with bandage. The process of wound healing was assessed by measurement of lesioned area after 3 days from the beginning of the application. Each column represents the mean ± SEM of the percentage of skin damage improvement. *$p < 0.05$ versus vehicle treated animals, $n = 8$ for each experimental group.

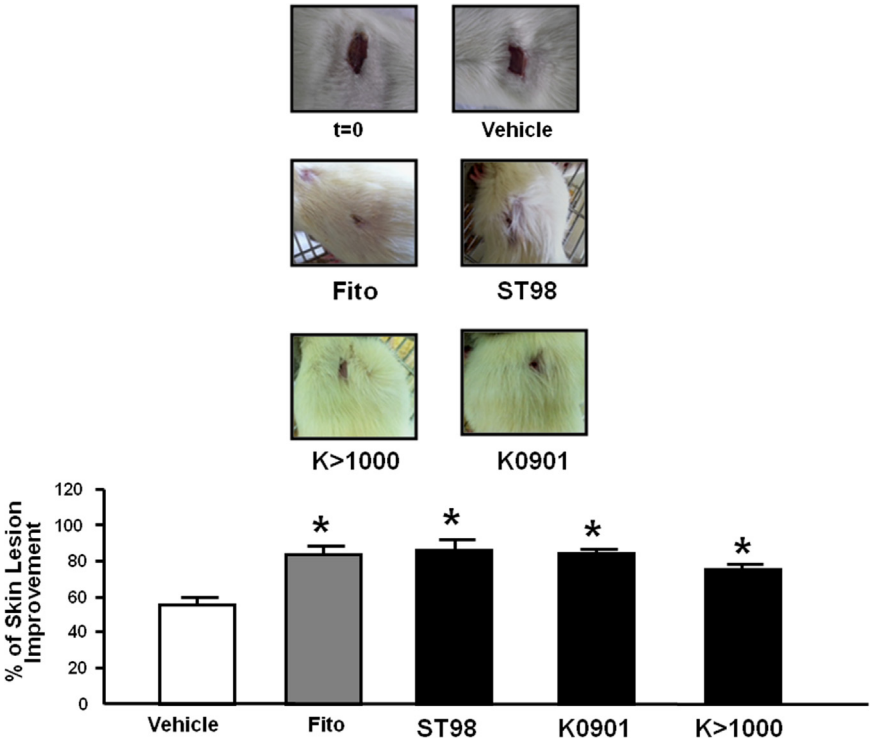

Fig. 7. Effect of fractions ST-98, K0901, K > 1000, on healing in the rat model of skin excision. The vehicle, or Fitostimoline® cream (P), or the fractions ST-98, K0901 and K > 1000 in a pharmaceutical form as a cream, were applied on the lesioned area daily, and the wound covered with sterile gauze was fixed with bandage. The process of wound healing was assessed by measurement of lesioned area after 7 days from the beginning of the application. Each column represents the mean ± SEM of the percentage of skin damage improvement. *$p < 0.05$ versus vehicle treated animals, $n = 8$ for each experimental group.

The fractions K > 1000 and ST-98 were then analyzed by the following MS approaches: (a) MALDI-TOF MS (MALDI Micro MX-Waters) and (b) ESI-QTOF-MS (QTOF Premiere-Waters) (see Section 2.5).

The MALDI-TOF mass spectrum of the fraction K > 1000 shows, along the *m*/*z* axis up to 3963.57 *m*/*z*, a Gaussian distributions of peaks differing for 162 uma, with a maximum at 1689.88 *m*/*z*.

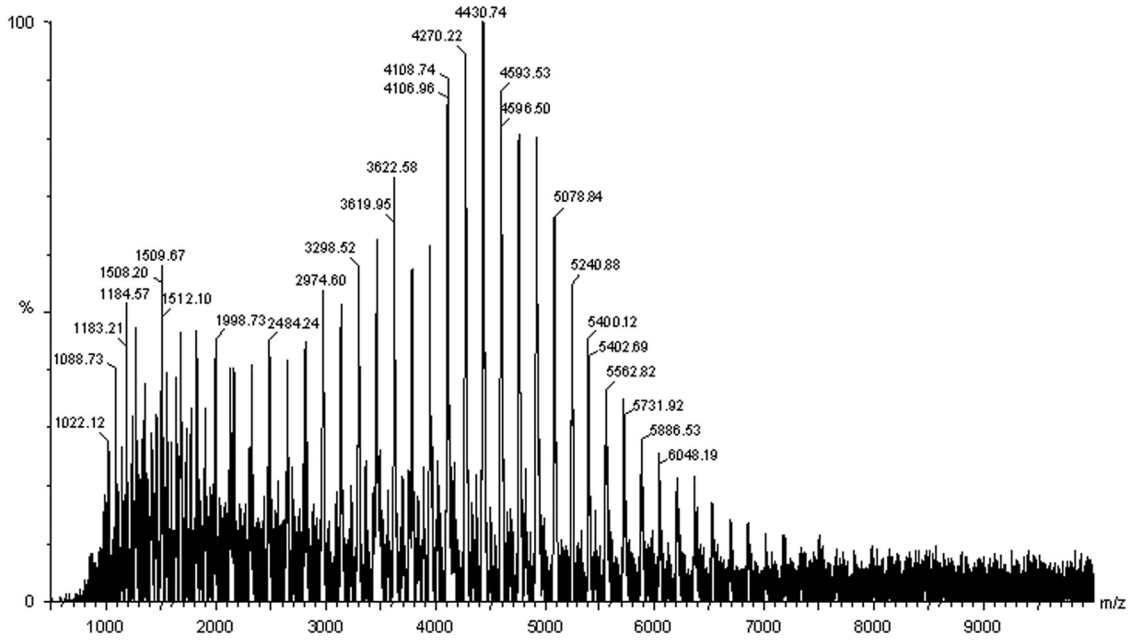

Fig. 8. Linear positive MALDI-TOF-MS obtained for fraction ST-98 using THAP matrix and laser energy at 250 arbitrary units.

The ESI-QTOF mass spectrum of fraction K > 1000 shows a Gaussian series of multi-charged peaks centered at 827.45 *m*/*z*. The deconvolution of the spectrum by Mass Lynx software establishes the presence of 31 oligomeric species differing by 162 uma in the MW range 1032.60–4962.90 Da. Some of the multi-charged ions from different oligomeric species were eventually selected for MS/MS fragmentation analysis. All fragmentation spectra of double-charged ions exhibited a sequential loss of 81 uma, that for $z=2$ indicates the sequential loss of an hexose fragment of 162 uma. Analogous results were obtained from fragmentation spectra of triple-charged peaks, that exhibited a sequential loss of 54 uma corresponding, for $z=3$, to an identical sequential loss of an hexose fragment.

The MALDI-TOF mass spectrum of the fraction ST-98 (Fig. 8) shows a Gaussian series of peaks differing by 162 uma up to 6045.02 *m*/*z* and centered at 4430.74 *m*/*z*.

The ESI-QTOF mass spectrum of fraction ST-98 shows, as for K > 1000, a Gaussian series of multi-charged peaks (Fig. 9). The deconvolution of the spectrum by Mass Lynx software (Fig. 9) establishes the presence of 18 oligomeric species differing by 162 uma in the MW range 3790.59–6547.98 Da. As for K > 1000, some of the multi-charged ions from different oligomeric species were eventually selected for MS/MS fragmentation analysis. All fragmentation spectra of double-charged ions ($z=2$) exhibited a sequential loss of 81 uma, while triple-charged peaks ($z=3$) exhibited a sequential loss of 54 uma, both corresponding to the sequential loss of an hexose fragment.

All the data from MALDI and ESI MS experiments are coherent and, combined with sugar analysis, indicate that both fractions K > 1000 and ST-98 are mainly constituted by a mixture of malto-oligosaccharides, generated from seed starch during the germination process. The fraction ST-98, that gives rise to the major HPAE peak eluting between 42 and 52 min (Figs. 1 and 2), is constituted by a restricted series of oligomers within the molecular weight range ca. 3800–6550 Da, while the fraction K > 1000 also contains consistent amounts of lower molecular weight congeners in the range ca. 1000–4000 Da.

4. Discussion and conclusions

The present paper describes a pro-proliferative effect of the fractions ST-98 and K > 1000 of TVE in in vitro and in vivo models of skin lesion; thus indicating the active fractions of TVE responsible for its pro-proliferative effect.

The aqueous extract of *Triticum vulgare* possesses a number of biological properties including a mitogenic effect both in plants and in the mouse fibroblast line BALB/c NIH-3T3 (Farinella et al., 1986; Favit et al., 1992), indicating potential properties of this extract as a trophic factor. Over the years there have been many studies that allowed the isolation and partial characterization of fractions derived from *Triticum vulgare* extract (TVE) (Vanden Berghe et al., 1993); these studies have demonstrated the ability of fractions to stimulate and promote cell proliferation of several cell lines such as human umbilical vein endothelial cells, fetal bovine aortic endothelial cells and baby hamster kidney cell clone 21.

In this study, we evaluated the effects of TVE and several fractions obtained from TVE on the proliferation of mouse fibroblast NIH-3T3 cells with the aim of identifying fractions able to maintain the healing activity of TVE. We specifically looked at the effects of the TVE and different fractions on cell growth in the experiments of proliferation and scratch tests. The results obtained in vitro showed a pro-proliferative effect of the fractions ST-98, and K > 1000, comparable to that of the entire extract (batches K0801 and K0901). As shown by sugar and mass spectra analyses, these fractions contain – in different amounts – a mixture of malto-oligosaccharides with molecular weights in the range 1000–6550 Da that gives rise to the major HPAE peak eluting between 42 and 52 min (Figs. 1 and 2). Moreover, as the Fitostimoline® cream based on TVE is widely used for its stimulating properties on wound healing, we decided to study the effects of the same fractions obtained from TVE and tested positive in laboratory studies on mouse fibroblasts. For this purpose, these fractions were developed as a cream containing as active ingredient the different fractions, and then we observed their effects on tissue regeneration and wound healing in two different models of skin lesion, scarification and excision of skin in rats. The results obtained in animal models were consistent with those obtained on murine fibroblasts, confirming the ability of the different fractions to stimulate the regeneration of the skin lesions. In particular, cream preparations obtained from the fractions ST-98 and K > 1000 showed a positive effect comparable to that of the commercial product Fitostimoline® cream.

In conclusion, our results demonstrated that we have isolated some of the active components of the extract of *Triticum vulgare* which, by themselves, retain their ability to stimulate cell

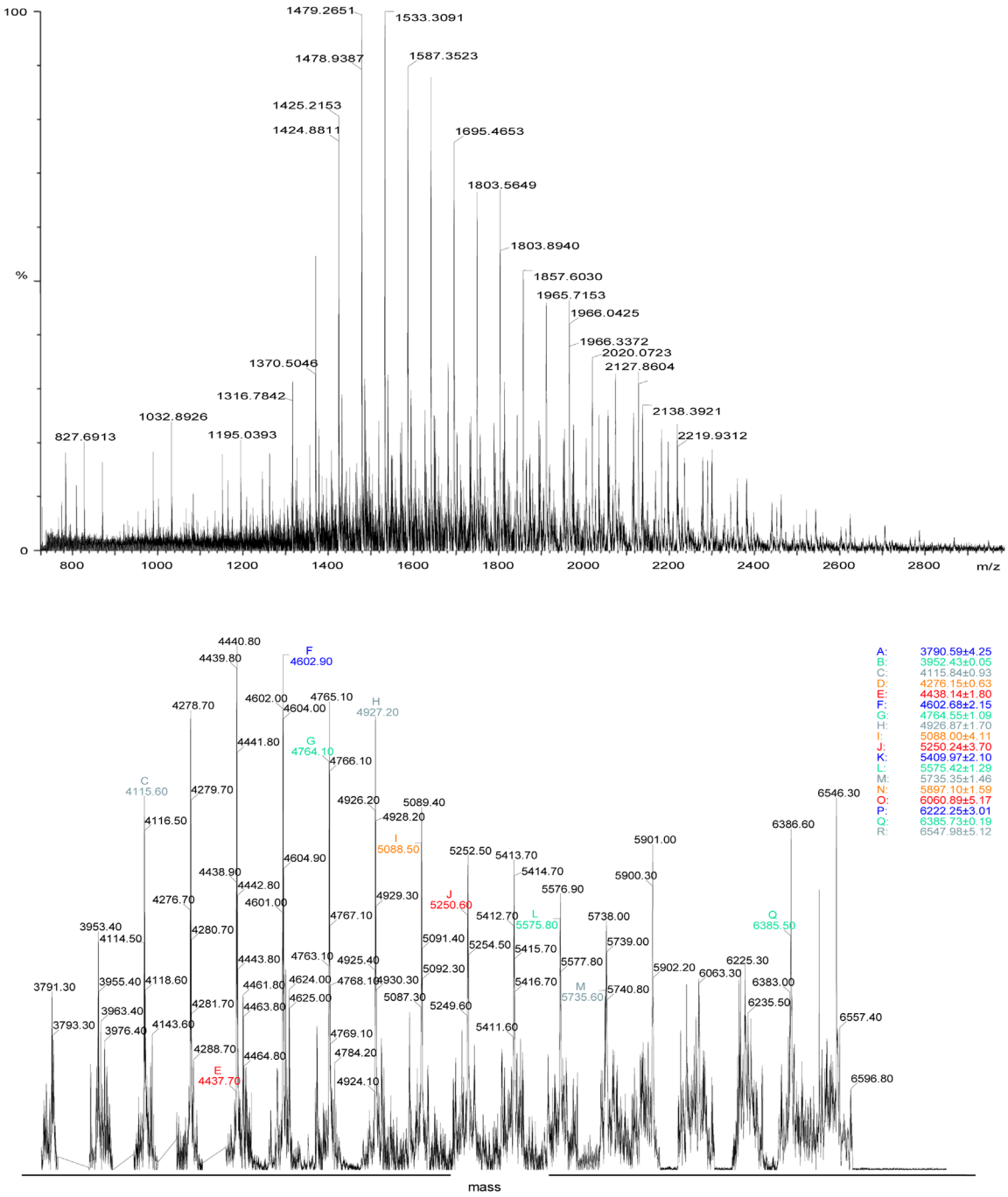

Fig. 9. The upper panel shows the multi-charged negative-ESI-TOF-MS spectrum obtained for fraction ST-98 diluted in water/30% isopropanol/2% tri-ethyl-amine. The lower panel shows the deconvoluted spectrum obtained by MassLynk 4.0 software together with the molecular weights of the species.

proliferation when tested on cultures of mouse fibroblast NIH-3T3 cells. These fractions showed similar and overlapping efficacy in stimulating and enhancing the healing of wounds in rat models of skin scarification and excision. The effectiveness of these fractions in stimulating tissue regeneration was comparable with that of the whole extract both in fibroblasts and in animal models.

Acknowledgments

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